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A Surface Acoustic Wave Biosensor Concept with Low Flow Cell Volumes for Label-Free Detection

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Special surface acoustic wave (SAW) devices using horizontally polarized surface shear waves can be operated in water. They allow an easy detection of molecules with biological relevance (e.g., proteins) via direct detection of the adsorbed mass. The transducer structures of conventional SAW devices are usually connected to the electronics by bond wires. In consequence, flow cell volumes can hardly be designed smaller than 50 μL. A new type of SAW device that is coupled capacitively with the electronics enables the reduction of flow cell volumes down to 60 nL, which decreases sample consumption and reduces the length of the measurement cycles down to a few minutes. To create an immunosensor, the SAW devices first are coated with a thin parylene layer for creating a sensor surface that is chemically homogeneous. Then OptoDex, a dextran containing both photoactive and functional groups is immobilized photochemically. Finally, antibodies are coupled via conventional EDC/NHS chemistry. The technique has been used to monitor urease binding at anti-urease-coated SAW devices in real time and with good resolution. Because of the simple sensor handling and the economical sample use, the new SAW device is particularly suitable for the design of an array.

Affinity assays require the transformation of the binding between ligand (e.g., antigen) and receptor (e.g., antibody) into a measurable quantity. Labeling the binding partners could alter their properties; thus, for evaluating the binding potential of biomolecules, unlabeled compounds should be used. All approaches reported in label-free biosensing so far are based on a heterogeneous test format. They allow the direct monitoring of molecular interactions on the transducer surface on-line and in real time.[1]

Surface acoustic wave (SAW) detection proved to be a sensitive and cost-effective method for label-free detection of biomolecular interactions. Binding reactions on the surface are detected by determining changes in surface wave velocity caused mainly by mass adsorption or viscosity changes in the sensing layer.[2]

Rayleigh wave devices commonly used for gas-sensing applications cannot be used for sensing in the liquid phase. The reason for this is a too high attenuation of the surface acoustic waves: due to their conversion into compressional wave components, acoustic energy is leaking from the surface into the bulk of the liquid phase. Thus, for a suitable operation in liquids, another special type of SAW device has to be used, allowing so-called horizontally polarized surface shear waves (HPSSW) or surface transverse waves.[3]

For biosensing, the high dielectric constant of the medium water ($\epsilon_r \approx 80$) has to be considered. Otherwise a significant electrical impedance mismatch leading to a weak acoustoelectric coupling would be observed. Thus, substrates with low dielectric constants (e.g., quartz, $\epsilon_r \approx 4.7$) can only be operated as Lamb wave devices[4] or as Love wave devices, i.e., after coating with an acoustically thick wave-guiding layer.[5] The transducers of the latter are typically further shielded by an additional layer preventing close contact of the medium water with the transducer area. Another way to cope with this problem without taking additional steps is the use of a substrate material with a sufficiently high dielectric constant such as lithium tantalate ($LiTaO_3$, $\epsilon_r = 43$). This material can be used for SAW biosensing without additional wave-guiding or transducer-shielding layers.[6,7]

However, the standard and low-cost material for transducers of SAW devices is aluminum. If one likes to use this cost-efficient setup for biosensing applications, these devices must indeed be coated with a shielding layer, but for another reason: preventing corrosion effects on the aluminum structures. Aromatic polyimide layers and poly-(2-chloro-*p*-xylylene) ("parylene C") were already successfully applied as suitable protection layers for SAW biosensors.[8,9] However, the aluminum structures are usually connected to the electronics via bond wires. In consequence, such SAW-based biosensor systems suffer from the fact that flow cell volumes can hardly be designed smaller than 50 μL. In addition,

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air bubbles tend to develop around the bond wires and may interfere with the measurements.[10]

In this work, we introduce a new type of SAW device with gold transducers on lithium tantalate substrates. Bond wires are eliminated as the sensor is coupled capacitively with the electronics; thus, sensor replacement is simplified. The new capacitive coupling concept enables the reduction of the sample volume in the flow cell. Furthermore, the thickness of the diffusion layer is minimized by using a small channel depth. Both measures led to a remarkable decrease of sample consumption and a reduction of response time.

Using gold transducers would make redundant the use of shielding layers to prevent corrosion effects. On the other hand, an additional polymer layer offers two other features that might improve the sensor characteristics: first, a suitable polymer layer can serve as wave-guiding layer as well, to tune the mass sensitivity of the sensor. If the layer thickness is chosen properly, the guiding layer will trap the acoustic energy near the surface, similar to the generation of Love waves (correctly defined only on devices with bare surface areas such as delay lines), leading to an increase in sensor response.[11] For a wave-guiding effect, the coating has to be uniform, well adhesive, and nonconductive. Moreover, it has to show low levels of intrinsic attenuation. Materials used as wave-guiding layers include sputtered SiO_2,[12] poly(methyl methacrylate)[13] and parylene C.[14] Second, the polymer surface of this layer represents a chemically homogeneous surface, which improves the success and reproducibility of further modification steps.

In this approach, parylene C was chosen as homogenizing polymer. Parylene C is known to generate extremely uniform and nonporous thin films with good adhesion to the substrate. It can be deposited with good reproducibility at room temperature in a vacuum chamber.[15] The use of a quartz microbalance enables easy control of the layer thickness during the deposition process.[14]

To create a biosensor, the receptive molecules are coupled via an intermediate dextran layer on the parylene C-coated SAW transducers. This circumvents binding of receptors directly on the parylene surface, which would require aggressive chemical conditions. The intermediate dextran layer enables the experimentally facile, rapid, and reproducible immobilization of a great variety of receptive molecules with established biochemical coupling methods. That implies mild reaction conditions, so that the receptors will keep their functionality.[16]

Dextrans can be bound covalently to the polymer layer by means of photoimmobilization.[17] This was applied successfully on SAW biosensors by single-step reaction of a copolymer consisting of carboxymethylated dextran and bovine serum albumin (BSA), whereas BSA was polyderivatized with light-sensitive trifluoromethyl aryldiazirine groups.[18] In this work, a dextran containing both photoactive and functional groups, OptoDex, was used to give the intermediate layer.[19] After binding OptoDex via photoreaction on the surface, the functional groups were used to immobilize receptors covalently via conventional EDC/NHS chemistry on the dextran surface. The last step can be performed on-line in order to control the efficiency of this modification.[9]

The dextran layer serves other purposes yet. All label-free detection methods suffer from the fact that each molecule binding on the surface is detected, whether the reaction is specific or not. Therefore, the biochemically active layer must not only enable specific interactions but must also prevent unspecific binding on the surface. Hydrogels such as dextran have been proven to be a good shielding layer against unspecific interactions.[20]

The three-dimensional structure of the dextran hydrogel matrix results in a high receptor density and a high binding capacity of the sensor. Earlier SAW experiments showed that a three-dimensional receptor layer increases sensitivities by 2 orders of magnitude compared to a two-dimensional receptor layer.[18] In addition, the high binding capacity enables mass-transport-limited binding of ligand molecules on the surface up to ~30% of the maximum surface coverage. Under these conditions, the binding rate is constant, which leads to a linear binding curve with direct detection methods. With constant flow conditions, the slope of the binding curve is a measure for the ligand sample concentration.[21]

EXPERIMENTAL SECTION

SAW Device and Instrumentation. New resonator filters based on HPSSW were designed in cooperation with Siemens and later purchased as E062 from Epcos. The devices have gold transducers and work at an operating frequency of 433.9 MHz on 36°YX $LiTaO_3$ as substrate material.

Measurements were done in an oscillator circuit developed in-house with the SAW device as frequency-determining element. The HF signal of the oscillator was converted by use of a permanently oscillating reference resonator (433.9 MHz). The low-frequency differential signal (<20 kHz) was passed on to an electronic counter equipped with a field-programmable gate array.[22] The phase was set by selecting an appropriate drive voltage.[23]

Flow cells were designed as part of an electronic circuit board integrated in the oscillator unit. The sensors were coupled capacitively with the driving electronics via large contact pads on the sensor layout and on the surface of the circuit board, respectively. Wiring and contact pads were made by laminated copper. Two types of flow cells were designed. The first had a milled flow channel with the dimensions 4 mm × 1.2 mm × 1 mm corresponding to a flow cell volume of 4.8 μL. Stainless steel capillaries, inner diameter 0.9 mm, were used as connections to the fluidics. In the second flow cell, a PTFE tube with an inner diameter of 0.2 mm was sliced at the sensor position. Thus, the

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tube served both as connection to the fluidics and as fluid channel itself, resulting in a flow cell volume of 60 nl.

In the beginning of each measurement, the frequency value on the monitor was reset to zero. Changes in the resonance frequency of the oscillator circuit were monitored continuously with a time resolution of 1 s. As differential frequencies (relative to the reference sensor) were used as signal output, processes leading to frequency decrease in the oscillator circuit, such as viscosity decrease and mass increase, resulted in increasing frequencies on the monitor.[24,25] The frequency resolution was 1 Hz. The short-term noise was determined to 40 Hz.

Chemicals. All chemical and biochemical products were of analytical grade. BSA was received from Serva Feinbiochemica GmbH (Heidelberg, Germany). Urease and monoclonal anti-urease were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Parylene C was obtained from Speedline Technologies.

Phosphate buffer contained 20 mM sodium dihydrogen phosphate. Phosphate-buffered saline (PBS) contained 20 mM sodium dihydrogen phosphate and 140 mM sodium chloride. The respective pH values were adjusted with KOH to 7.2. Acetate buffer was prepared by titrating 10 mM acetic acid to pH 5 with KOH solution.

OptoDex-A (i.e., dextran with photoactive and amino groups) was prepared according to Caelen et al.[19]

Preparation of Sensor Surfaces, Samples, and Carrier Stream. All SAW devices were cleaned by rinsing with 2-propanol and drying with cotton swabs before further use.

(1) Adsorption Tests and Viscosity Tests. In both kinds of measurements, uncoated SAW devices were used. For *adsorption tests*, samples were prepared by dissolving 4 mg of BSA/mL of phosphate buffer. Phosphate buffer was used as carrier stream. For *viscosity tests*, samples were prepared by adding 0, 12.5, 25, 37.5, or 50 vol % ethylene glycol to bidistilled water. The viscosities (T = 22 °C) were calculated from tabulated values to 0.94, 1.35, 1.89, 2.65, or 3.75 mPa·s.[26] Bidistilled water was used as carrier stream.

(2) Immunoassays. Parylene C was deposited via chemical vapor deposition at low pressure and room temperature with a commercial device (Labcoter 1, Parylene Deposition Unit model PDS 2010; Speedline Technologies) equipped with a QMB unit for monitoring the parylene thickness during deposition. SAW devices coated with 0.1 μm of parylene were incubated with a solution of 0.1 mg/mL OptoDex-A in PBS according to Caelen et al.[19] (In this particular step, PBS containing 50 mM sodium phosphate and 150 mM sodium chloride was prepared, adjusted to pH 7.4, and diluted with bidistilled water 1:100.) After drying for 3 h in a vacuum, the surfaces were irradiated for 4 min in a Stratalinker 2400 UV Crosslinker (Stratagene GmbH, Heidelberg, Germany) equipped with five UV light bulbs (365 nm major emission, 15 W each). After photobonding, the surfaces were washed with PBS containing 0.05% (v/v) Tween 20, PBS, and bidistilled water. Each washing step was repeated 3 times and included intermittent shaking for 5 min. The amino groups were converted to carboxyl groups via 16-h reaction with a 2 mg/μL solution of glutaric anhydride in dimethyl formamide. Anti-urease was immobilized on-line according to Barié et al.[9] PBS was used as carrier stream. First the surface was activated for 10 min with a solution of 0.05 M *N*-hydroxysuccinimide and 0.2 M *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) in bidistilled water and rinsed for 5 min with PBS. Then the surface was incubated for 20 min with a solution of 0.01 mg/mL anti-urease in acetate buffer and rinsed for 5 min with PBS. After that, remaining reactive groups on the surface were deactivated for 10 min with a solution of 1 M ethanolamine, pH 8. After equilibration by rinsing with buffer, the sensor surface is ready for immunoexperiments. BSA in PBS, c = 4 mg/mL, and urease in PBS, c = 25 and 250 μg/mL, respectively, were used as samples. PBS was used as carrier stream.

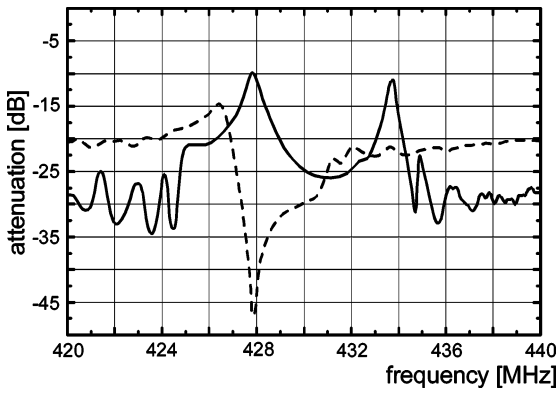

Figure 1. Transmission curve of an uncoated E062 SAW device in air (solid line) and in bidistilled water (dashed line).

Sample Handling. Samples were handled with a self-constructed flow injection analysis system equipped with two peristaltic pumps (Ismatec, Wertheim, Germany), a six-way valve (Besta-Technik, Wilhelmsfeld, Germany) for loading samples into the sample loop, and an injection valve (Besta-Technik) for switching between load and inject modes. PTFE tubes served as connections between single units and as sample loop. During the inject mode, the content of the sample loop was driven to a flow-through SAW detector. The flow directions in the sample loop during loading and injection were opposite to each other in order to reduce dispersion of the sample. The flow rate was adjusted to 120 μL/min during the measurements.

RESULTS AND DISCUSSION

Sensor Characteristics and Measurement Options. The solid line in Figure 1 represents the transmission curve of an uncoated E062 SAW device in air. As is characteristic for resonance filters, the transmission curve shows two maximums, one at 427.9 MHz (−9.7 dB) and the other at 433.7 MHz (−10.9 dB). The dashed line in Figure 1 represents the transmission curve of the same uncoated E062 SAW device in bidistilled water. In water, one of the maximums, the parallel resonance, disappears due to the changed conditions of the dielectric environment in water. The remaining serial resonance at 426.5 MHz represents the real acoustic mode based on a HPSSW transmission with an attenuation of −14.7 dB. This value is remarkably low and can easily be handled by using commercial low-noise HF amplifiers.

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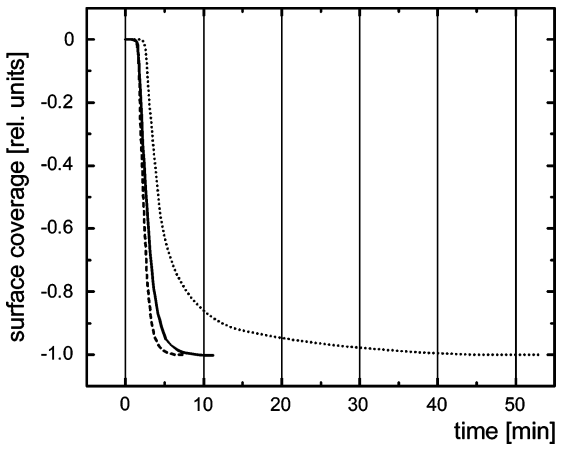

Figure 2. Surface coverage during BSA adsorption on a SAW device in a flow cell with volumes of 50 μL (dotted line), 4.8 μL (solid line), and 60 nL (dashed line).

For this reason, a good signal-to-noise ratio (S/N) for in situ bioanalytical detection with the sensor immersed in water or buffer solutions is ensured without the necessity of using additional wave-guiding layers.[7] Due to the gold transducers, a shielding layer to prevent corrosion effects is not necessary any more.

Figure 2 shows the surface coverage as a function of time during BSA adsorption on uncoated SAW devices in three types of flow cells (see Adsorption Tests and Viscosity Tests). The dotted line represents the surface coverage obtained with a previously used SAW sensor configuration. These sensors were connected with bond wires via a socket (standard TO39 cases) to the electronics, which required the use of a comparably large flow cell volume of 50 μL.[9] Solid and dashed lines represent the surface coverages on the E062 SAW devices in newly designed flow cell setups where the devices are coupled capacitively to the electronics via large contact pads (see SAW Device and Instrumentation). This enabled downsizing of the flow cell volumes to 4.8 μL (solid line) and to 60 nL, respectively. The reduction of the flow cell volumes reduced the duration of the measurement cycles from more than 0.5 h (flow cell volume 50 μL) down to a few minutes (flow cell volumes 4.8 μL and 60 nL, respectively). However, the time reduction from the 4.8-μL cell to the 60-nL cell was comparatively low. This may be due to the valves and the tubing length of the remaining fluidic.

Figure 3 shows the behavior of two uncoated E062 SAW sensors treated with solutions of different viscosities. Samples with volume concentrations in a range from 0 to 50% ethylene glycol in bidistilled water, representing a viscosity range from 0.94 to 3.75 mPa·s (T = 22 °C), were injected into a bidistilled water carrier stream (see Adsorption Tests and Viscosity Tests). Increasing viscosities led to decreasing difference frequencies. The values of maximum frequency shift were taken for evaluation (Figure 4). For each sensor, three series of measurements were carried out and subsequently averaged. Standard deviations are represented by error bars. The slopes of the regression lines were -6.0 ± 0.6 kHz/vol % for sensor 1 (solid squares, solid line) and -6.8 ± 0.6 kHz/vol % for sensor 2 (open circles, dotted line), which means they matched considering the standard deviation ranges. The reproducible results in this viscosity range demonstrated that the sensor can be used for common protein samples. Human blood serum, e.g., has a viscosity of 1.27 mPa·s (T = 37 °C);[27] thus, the viscosity of diluted protein solutions at T = 22 °C would be in the range from 0.94 mPa·s to maximal 1.27 mPa·s.

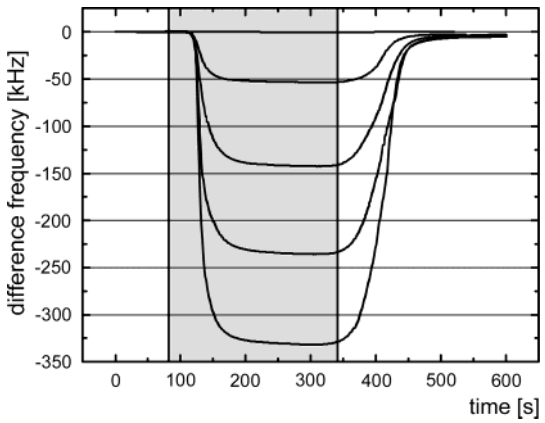

Figure 3. Difference frequency values monitored with samples of different viscosities on an uncoated SAW device. Carrier stream, bidistilled water. Samples (from top): 0, 12.5, 25, 37.5, and 50 vol % ethylene glycol in bidistilled water; this corresponds to viscosities of (from top, T = 22 °C) 0.94, 1.35, 1.89, 2.65, and 3.75 mPa·s. The injection interval is marked gray.

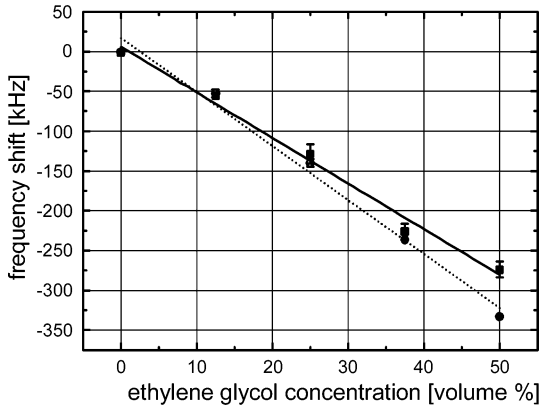

Figure 4. Difference frequency shifts of two uncoated SAW devices in the viscosity range 0.94−3.75 mPa·s. Carrier stream and samples, see Figure 3. Each concentration was measured once in a series of measurements; three series were taken. Straight and dotted lines represent the regression lines.

SAW Biosensing. To create an immunosensor, anti-urease was coupled on a sensor coated with 0.1 μm of parylene C, OptoDex-A, and glutaric anhydride (see Immunoassays). Sensor modification with parylene C, OptoDex-A, and glutaric anhydride was done off-line. Binding of anti-urease was carried out on-line in order to control the success (Figure 5). The large frequency shifts during the injection intervals were based on the change between ionic strength of the reactant solutions and the carrier buffer. Thus, the real frequency change due to covalently bound antibody cannot be estimated until the end of the measurement when the surface is rinsed again with carrier buffer. After all preparation steps, the overall difference frequency shift compared to the initial resonant frequency was 43 kHz, representing the amount of covalently bound receptor molecules within the dextran matrix. The positive signal shift demonstrated that the effect of mass increase prevailed over the effect of the increase of viscoelasticity.

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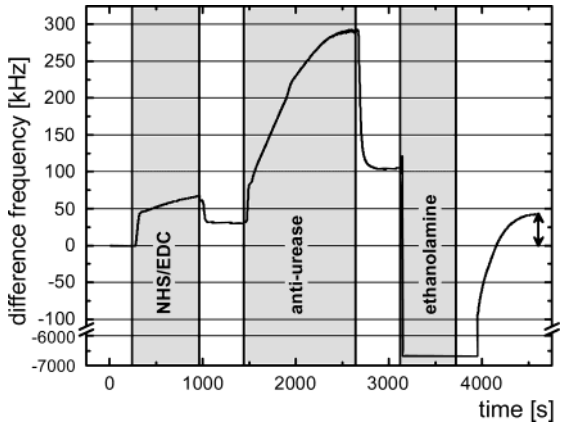

Figure 5. On-line modification of an E062 SAW device coated with 0.1 μm of parylene C and OptoDex. Receptor to be immobilized, anti-urease. Carrier stream, PBS. The injection intervals of the reactants are marked gray.

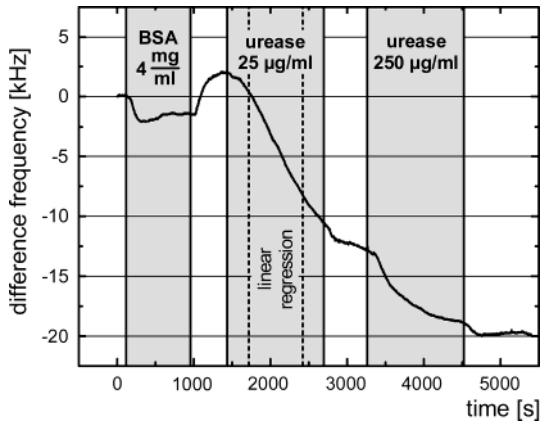

Figure 6. Immunoassay with an E062 SAW sensor modified according to Figure 5. Sample solvent and carrier stream, PBS. The injection intervals of the reactants are marked gray. The dashed lines indicate the linear regression interval.

After the surface was modified with anti-urease, the sensor was used for immunoreaction (Figure 6). Unspecific interactions were observed by incubating the surface with 4 mg/mL BSA. The frequency shift during incubation was reversible, so it can be explained by the higher viscosity of the BSA solution compared to the carrier stream. The higher difference frequency after rinsing, compared to the frequency at the beginning of the measurement, was based on mass increase due to adsorbed BSA. The signal shift of 2 kHz is negligible considering the difference frequency shifts obtained with the specific antigen urease. So the surface shielding with the dextran derivative OptoDex-A was successful.

When the sensor was incubated with 25 μg/mL urease, the binding curve decreased linearly for several minutes. Linear regression of this part of the binding curve resulted in a slope of -12.62 ± 0.02 Hz/s, which is significantly different from the drift of $+0.27 \pm 0.02$ Hz/s determined at the end of the measurement. The slope began to change only after a total difference frequency shift of -8.5 kHz was reached. After the surface was incubated with 250 μg/mL urease, the difference frequency of the equilibrium coverage was determined to -21 kHz. According to Piehler et al.,[21] a higher shift would have been expected, as the linear range is expected to be up to 30% of the maximum surface coverage. In fact, 250 μg/mL urease is a high concentration, but to determine the real saturation coverage, several samples with high urease concentrations had to be incubated on the sensor. This was not done, as the range of linearity was already determined.

Due to the 3D matrix obtained with OptoDex-A, the sensor had a high binding capacity to urease. Therefore, with shorter injection times and protein concentrations being commonly used in analytical tasks, the binding capacity is high enough to determine several protein concentrations on one sensor, without regeneration steps, via the slope of the binding curve. Binding urease leads to mass increase on the surface and to increasing rigidity in the antibody-modified dextran layer. The difference frequency signal decreased, so the effect of viscoelasticity prevailed over the effect of mass increasing. Rinsing did not remove the urease; the binding was irreversible.

The capacitive coupling concept required reproducible clamping conditions. Otherwise the sensor response was influenced not only by the sample composition but also by two other effects: first, variations in the depth of the flow channel influenced the thickness of the diffusion layer, leading to different slopes of the binding curve despite constant sample concentrations. Second, additional forces stressing the substrate material had an effect on the propagating acoustic surface wave and influenced the output frequency as well. With constant clamping conditions, signal deviations below 5% were obtained.

CONCLUSION

The newly designed E062 SAW device consisting of lithium tantalate substrate with gold transducers showed excellent sensing properties compared with other sensor devices of our own[2] or other groups' [28] previous approaches. As the transducers consist of gold, shielding layers for preventing corrosion effects are not necessary. The remarkably low insertion attenuation of -14.7 dB in water can be easily handled by using commercial low-noise HF amplifiers ensuring a good signal-to-noise ratio in aqueous buffer solutions without using additional wave-guiding layers. However, this does not mean that such layers cannot be used for enhancing the signal characteristics of the sensor, which will be shown in future experiments.

The SAW devices were coupled capacitively with the electronics, which prevents the use of bond wires and thus enabled reducing the flow cell volume down to 4.8 μL for standard applications and to 60 nL for applications where extremely low sample consumption is required. The duration of the measurement cycles was significantly reduced from more than half an hour to a few minutes.

Measurements with aqueous ethylene glycol solutions demonstrated that the results were reproducible at least in a viscosity range from 0.94 to 3.75 mPa·s ($T = 22$ °C), which is adequate for common protein samples.

OptoDex-A proved to be both an excellent coupling agent for binding antibodies on parylene and a good shielding layer against unspecific interactions. The sensor with immobilized anti-urease had a high binding capacity to urease due to the 3D matrix obtained with OptoDex-A, which enabled the determination of urease concentrations within a wide concentration range. Still, unspecific interactions resulting from BSA adsorption were suc-

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cessfully minimized. The experiments could be repeated with good reproducibility.

Because of the simple sensor handling and the economical sample use, the new SAW device is particularly suitable for the design of an array. The capacitive coupling of the E062 SAW sensor device simplifies sensor replacement, even without socket. So an array for SAW biosensing, analogous to our SAW gas-sensing array,[29] will be accomplished soon.

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